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Identification of RTX toxin target cell specificity domains by use of hybrid genes.
Forestier C; Welch RA
Department of Medical Microbiology and Immunology, University of Wisconsin-Madison 53706.
Infect Immun (UNITED STATES) Nov 1991, 59 (11) p4212-20, ISSN 0019-9567 Journal Code: GO7
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Languages: ENGLISH
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Comparison of the toxic and antigenic properties of single bovine isolates of Pasteurella haemolytica representing five serotypes and an untypable strain.
Gentry MJ; Confer AW; Holland SG
Department of Veterinary Parasitology, Microbiology and Public Health, Oklahoma State University, Stillwater 74078.
Vet Microbiol (NETHERLANDS) Apr 1988, 16 (4) p351-67, ISSN 0378-1135 Journal Code: XBW
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Deletion analysis resolves cell-binding and lytic domains of the Pasteurella leukotoxin.
Cruz WT; Young R; Chang YF; Struck DK
Department of Medical Biochemistry and Genetics, College of Medicine, Texas A & M University, College Station 77843.
Mol Microbiol (ENGLAND) Nov 1990, 4 (11) p1933-9, ISSN 0950-382X Journal Code: MOM
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Comparison of the Toxic and Antigenic Properties of Single Bovine Isolates of *Pasteurella haemolytica* Representing Five Serotypes and an Untypable Strain*

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ABSTRACT

Gentry, M.J., Confer, A.W. and Holland, S.G., 1988. Comparison of the toxic and antigenic properties of single bovine isolates of *Pasteurella haemolytica* representing five serotypes and an untypable strain. *Vet. Microbiol.*, 16: 351-367.

Single strains of 5 different *P. haemolytica* serotypes (1, 2, 5, 6 and 9) and an untypable strain were compared in an attempt to detect differences which might be related to virulence. All but the untypable strain caused extensive lesions when injected into the lungs of healthy cattle. Each strain was found to be encapsulated and to be toxic in vitro for bovine leukocytes. Each strain also produced leukotoxin in vitro. The toxins varied, however, in total toxic activity and in the kinetics of leukotoxin production. Vaccination of cattle with each of the serotype strains elicited antibodies to organism somatic antigens and, to various degrees, the production of leukotoxin-neutralizing antibodies which showed no strain specificity in cross-neutralization studies. Although each of the serotype strains appeared to be a potential bovine pathogen, subtle differences were observed which may explain the importance of Serotype 1 strains in bovine pneumonic pasteurellosis.

INTRODUCTION

Pasteurella haemolytica causes pneumonia in cattle, sheep, goats and occasionally swine and causes septicemia in lambs and mastitis in ewes (Carter, 1968). To date, 15 different serological types of the organism have been differentiated by the indirect hemagglutination test (IHA) (Biberstein et al., 1960; Biberstein and Thompson, 1966; Pegram et al., 1979; Fraser et al., 1982a).

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Approximately 90% of *P. haemolytica* strains isolated from cattle and sheep can be grouped into these recognized serotypes, with the remaining strains collectively referred to as IHA negative or untypable (UT) (Donachie et al., 1984). Attempts have been made to identify serotypes among the IHA-negative strains with the conclusion that there may be as many as 9 different serogroups represented among the UT strains (Frank, 1980; Donachie et al., 1984).

All 15 serotypes of *P. haemolytica* plus several UT strains have been isolated from sheep (Mwangota et al., 1978). Serotype 2 is the most common isolate from healthy sheep (Biberstein and Thompson, 1966; Frank, 1982) as well as from cases of ovine pneumonic pasteurellosis (Thompson et al., 1977). Other serotypes cause pneumonia in sheep less frequently (Biberstein and Thompson, 1966; Fraser et al., 1982b). In contrast, Serotype 1 is the most common isolate from cases of the severe fibrinous pleuropneumonia associated with shipping fever of cattle (Wessman and Hilker, 1968; Fox et al., 1971; Mwangota et al., 1978; Allan et al., 1985). Serotype 2 is the most frequent isolate from the nasal passages of healthy cattle, but it is seldom isolated from cases of bovine pneumonic pasteurellosis (Fox et al., 1971; Frank and Smith, 1983).

Differences in frequency of isolation of *P. haemolytica* serotypes from natural cases of disease have led to the hypothesis that variations occur in virulence among the serotypes (Biberstein et al., 1960; Carter, 1963; Fox et al., 1971). However, when the pathogenicity of 2 common (Serotypes 1 and 2) and 2 less common (Serotypes 7 and 9) ovine isolates were compared, it was concluded that in sheep there were no clear differences in virulence among those serotypes (Gilmour et al., 1986). The purpose of the present study was to test the hypothesis that serotypes of *P. haemolytica* differ in their virulence for cattle because of differences in encapsulation, leukotoxin production or antibody response to the organism or its products.

MATERIALS AND METHODS

Microorganisms

Single isolates of 5 different serotypes and one UT strain of *P. haemolytica* were used in the study. The Serotype 1 (S1) strain was isolated originally from the trachea of a feedlot calf (Dr. R.J. Panciera, College of Veterinary Medicine, Oklahoma State University, Stillwater, OK). The organism was passed repeatedly in susceptible calves by intrapulmonic inoculation and re-isolation of the bacterium from the resulting pulmonic lesion. The Serotype 2 (S2), Serotype 6 (S6) and the untypable (UT) strains were bovine nasal isolates kindly provided by Dr. Glynn Frank (National Animal Disease Center, Ames, IA). The Serotype 5 (S5) and Serotype 9 (S9) strains were isolated by Dr. George Burrows (College of Veterinary Medicine, Oklahoma State University, Stillwater, OK) from tracheal swabs of feedlot cattle with symptoms of respiratory

disease. The serotypes of the S1, S5 and S9 isolates were determined in our laboratory by the rapid plate agglutination technique (Frank and Wessman, 1978) and were confirmed by Dr. Frank. The organisms were stored at −70°C in brain heart infusion (BHI) broth (Difco Laboratories) containing 15% glycerol (Sigma Chemical Co.). For each experiment, the frozen cultures were inoculated onto BHI agar supplemented with 5% bovine blood, 10% horse serum (Hazelton Dutchland, Inc.) and 1% yeast hydrolysate (ICN Pharmaceuticals). After incubation for 18–20 h at 37°C in a 5% CO_2 atmosphere, isolated colonies were chosen for further culture. Encapsulation of 6-h cultures of each strain was determined by the Maneval technique (Corstvet et al., 1982).

Experimental challenge

Approximately 5×10^9 CFU of each organism were injected into both lungs of a healthy 8–10-month-old calf by a trans-thoracic, intrapulmonic injection as described previously (Panciera and Corstvet, 1984). The severity of the resulting experimental disease was determined by a lesion scoring system based on the size and extension of lesions. Lesion scores of 0–20 were assigned with higher scores representing the more severe lesions (Panciera et al., 1984).

Leukotoxin production

Leukotoxin was prepared from each culture by the method of Shewen and Wilkie (1985). Briefly, organisms grown for 4.5 h to logarithmic phase in BHI broth were collected by centrifugation (13 500 × *g*, 20 min), resuspended in RPMI 1640 medum (Gibco Laboratories) supplemented with 7% fetal bovine serum (FBS, Hyclone Laboratories) and incubated for 1 h at 37°C. The cultures were then centrifuged and the supernatants, containing leukotoxin, were filter sterilized and frozen at −20°C. Viable bacterial cell counts were determined for each culture at the beginning and end of the 1-h incubation period by a spot plate method. In one experiment, toxin production by 4 of the 6 strains was allowed to continue for 8 h, well into stationary phase, during which culture supernatants were sampled for viable cell counts and leukotoxin quantitation at 1–2-h intervals.

To quantitate total toxic activity, extinction endpoints were determined for each toxic culture supernatant by a modification of a previously described ^{51}Cr-release method (Gentry et al., 1985a). Briefly, serial 2-fold dilutions (1/2–1/256) of the culture supernatants were made in RPMI 1640 medium in 96-well microtiter plates (Cell Wells, Corning Glass Works). The volume of diluted supernatant remaining in each well was 180 μl. Ninety microliters of a suspension of ^{51}Cr-labelled bovine peripheral blood leukocytes were added to each well and the plates were incubated for 1 h at 37°C in a 5% CO_2 atmosphere. The plates were then centrifuged at 200 × *g* for 10 min and 200 μl of

supernatant from each well were assayed for radioactivity in an automated gamma counter (Searle Analytical Co.). The leukocyte extinction endpoint was defined as the highest supernatant dilution eliciting a ^{51}Cr release of at least 50% of a total release control (4 N NaOH).

In vitro toxicity of organisms for bovine leukocytes

Mixed peripheral leukocytes were isolated from heparinized bovine blood and labelled with $Na_2(^{51}Cr)O_4$ (ICN Pharmaceuticals, Inc.) as previously described (Gentry et al., 1985a). The washed labelled cells were suspended in RPMI 1640 medium at a concentration of 6.0×10^6 cells ml^{-1}. Each bacterial culture to be tested was grown for 4.5 h in BHI broth at 37°C on a rotary shaker. The organisms were collected by centrifugation (13 500 × *g* for 20 min) and were resuspended in RPMI 1640 medium containing 7% FBS. Cultures were standardized to an optical density at 650 nm (LKB Ultraspec-4050 spectrophotometer, Bromme, Sweden) of 0.750 ± 0.005, with the exception of S9, which had an optical density of 1.233. The suspensions were found to contain $1.8–3.8 \times 10^8$ colony forming units ml^{-1} as determined retrospectively by a spot plate technique.

Four serial 10-fold dilutions of the organisms were prepared in serum-supplemented medium from each culture in duplicate. One hundred and eighty-microliter aliquots of each dilution prepared were placed in pentuplicate wells of 96-well tissue culture plates. Ninety microliters of the ^{51}Cr-labelled leukocyte suspension were added to each well and the plates were incubated for 1 h at 37°C in an atmosphere containing 5% CO_2. After incubation, the plates were centrifuged and the well contents were harvested and counted as in the extinction endpoint assay. Mean 4-min test counts from all 10 wells containing like dilutions of a given suspension were used to calculate the percentage killing of the leukocytes as follows:

$$\% \text{ killing} = [(\text{test count} - \text{medium control count})/(\text{total release control count} - \text{medium control count})] \times 100$$

Cattle vaccination and serology

Eighteen yearling crossbred male and female beef calves were allotted to 6 groups. Each group was vaccinated subcutaneously twice on Days 0 and 7 with 5 ml of a 22-h culture of live *P. haemolytica* organisms of a given serotype strain (Panciera et al., 1984). Each suspension was made in phosphate-buffered saline to an optical density at 650 nm of 1.600 ± 0.058. The average number of colony forming units per milliliter in the suspensions, as determined by retrospective plate counts, was 5.9×10^8 ml^{-1}. Blood samples were collected from each calf on Days 0 and 21.

Sera from the calves were tested for antibodies to somatic antigens from each

of the serotype strains using an enzyme-linked immunosorbent assay (ELISA). The assay was performed as described previously (Confer et al., 1985) with the exception that the antigens used to coat the plates were 22-h formalin-killed organisms (Confer et al., 1983).

The serum antibody response to a high molecular weight carbohydrate–protein subunit chromatographically separated from a saline extract of a 6-h culture of *P. haemolytica* Serotype 1 was also determined by ELISA as described previously (Confer et al., 1985). Antibody responses are expressed as the absorbance at 490 nm for unknown sera minus the absorbance for a phosphate-buffered saline control.

Leukotoxin neutralization (LN) titers were determined for the sera against leukotoxin prepared from each of the 6 serotype strains by a modification of a visual microtiter neutralization assay (Gentry et al., 1985a). Briefly, 2-fold serial dilutions of the sera were made in 96-well tissue culture plates, leaving 60 μl of diluted serum per well. One hundred and twenty microliters of leukotoxin were added to each well and the plates were incubated for 10 min at room temperature before 90 μl of a target cell suspension of freshly prepared bovine leukocytes was added. The plates were incubated for an additional 1 h, centrifuged at a low speed, and the cell pellets fixed, stained and examined. Leukotoxins used in the neutralization assay were standardized to an extinction endpoint of 1/16. Leukotoxin-neutralizing titers are expressed as the reciprocal of the last serum dilution that neutralized the toxin.

Statistical analysis

Analysis of variance was performed on values from the leukocyte toxicity assay. For treatment groups in which P was <0.05, differences in treatment means were compared by the least significant difference test (SAS Institute, Inc., 1982).

RESULTS

Experimental challenge

When injected into the lungs of calves, each of the serotype strains produced pneumonic lesions. The calf receiving the S1 strain had a lesion score of 12. The calves receiving the other serotype strains were scored as follows: S2 = 13; S5 = 20 (calf died 10 h after challenge); S6 = 18; S9 = 15. The calf injected with the UT strain had minimal lesions as evidenced by a lesion score of 3.

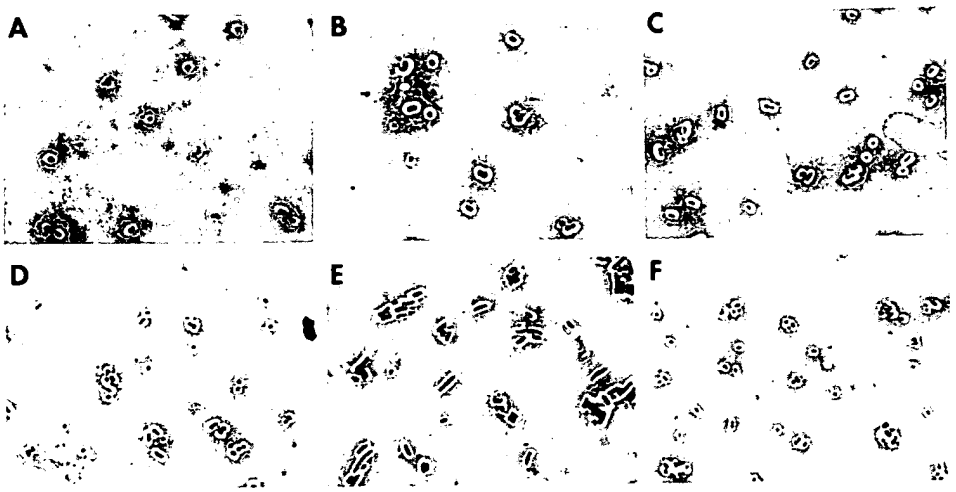


Fig. 1. Six *P. haemolytica* serotype strains stained by the Maneval method (×900) after growth for 6 h on supplemented BHI medium. (A) Serotype 1; (B) Serotype 2; (C) Serotype 5; (D) Serotype 6; (E) Serotype 9; (F) untypable strain. Organisms from all strains appear to be encapsulated.

Cultural characteristics and encapsulation

When plated on BHI agar supplemented with bovine blood, horse serum and yeast hydrolysate, all 6 serotype strains were hemolytic to slightly varying degrees. Cultures of S6 were distinguishable from the others due to their mucoid nature, with colonies that appeared to flow together rather than remaining discrete. Capsule stains performed on 6-h culturesof each strain showed that all were encapsulated (Fig. 1). The organisms were indistinguishable on gram stains (not shown) and the capsule stain, with the exception of S9. The organisms from the latter strain were much longer on average and could not be considered coccobacilli (Fig. 1E).

Leukotoxin production

Extinction endpoints of leukotoxins produced by 3–5 replicate cultures of each serotype strain ranged from 1/8 to 1/512 (Table I). The mean percentage increase in number of viable organisms in the cultures during toxin production was comparable for the S1, S5, S6 and S9 strains, but was only 1/3rd to 1/6th as great for the S2 and UT strains.

Eight-hour growth curves and leukotoxin measurements were determined for the S1, S2, S9 and UT strains (Fig. 2). The number of viable organisms in all 4 cultures increased during the first hour of incubation. The organisms continued to increase in numbers for the S1 and S2 strains, reaching a plateau

TABLE I

Comparison of percent viable cell increases and extinction endpoints of leukotoxins produced by 6 strains of *P. haemolytica*

Strain serotype	Number of replicates	Percent increase in CFU ml^{-1} [a]		Leukotoxin extinction endpoints[b]	
		Range	Mean	Range	Geometric mean
S1	5	85-224	153	1/16-1/128	1/42.2
S2	5	17-27	23	1/8-1/16	1/10.6
S5	5	147-182	168	1/16-1/64	1/32.0
S6	5	127-200	155	1/16-1/64	1/42.2
S9	5	110-190	152	1/64-1/512	1/147.0
UT[c]	3	47-65	56	1/8-1/32	1/16.0

[a]Percentage increase in number of colony-forming units per milliliter during the 1-h incubation period of toxin production. Calculated as: (final viable cell count − initial viable cell count/initial viable cell count) × 100.
[b]Extinction endpoints expressed as the highest dilution of leukotoxin causing a ^{51}Cr release from labelled bovine leukocytes of at least 50% of a total release control (4 N NaOH).
[c]Untypable strain.

after 2 and 6 h of incubation, respectively. The viability of the S9 and UT strains drastically decreased after the first hour of incubation.

Toxicity of the supernatant from the S1 and S2 strains was greatest at 2 and 4 h, respectively, followed by a gradual decline (Fig. 2A and B). Supernatants from the S9 and UT cultures had extinction endpoints of 1/16 and 1/32, respectively, when sampled at Time 0. Toxicity of the S9 strain was greatest at 2 h and remained high (1/32-1/64) for the remainder of the sampling period (Fig. 2C). Toxicity of the UT strain peaked at 1 h and then rapidly declined (Fig. 2D).

Toxicity of organisms for bovine leukocytes

When leukocytes were exposed to each of the 6 serotype strains at 4 different dilutions, there were slight variations in toxicity among the cultures, some of which proved to be significantly different (Table II). If the strains are arranged in decreasing order of the amount of ^{51}Cr released, S9 was the most toxic at all 4 dilutions and S1 was the least toxic at 3 of the 4 dilutions tested. The suspension of the S9 strain, however, ranked lowest of all the cultures in the number of viable organisms per milliliter (Table II).

Serologic evaluation of vaccinated cattle

Vaccination with each of the serotype strains induced cross-reacting antibodies to several of the other strains tested (Table III). Notable exceptions

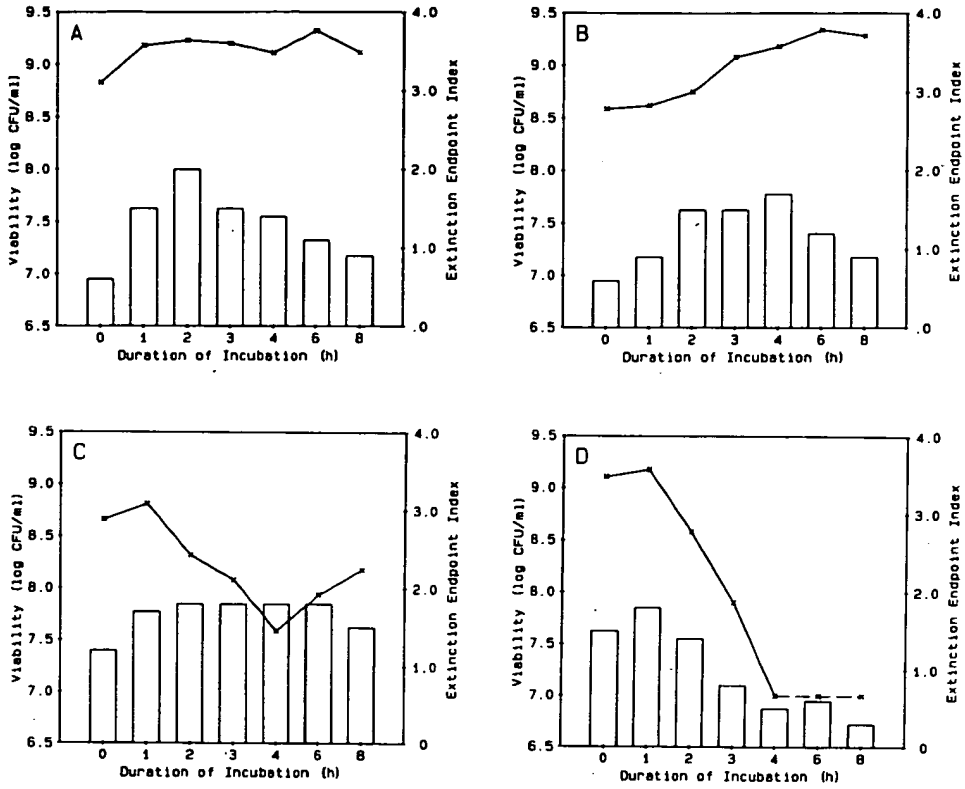
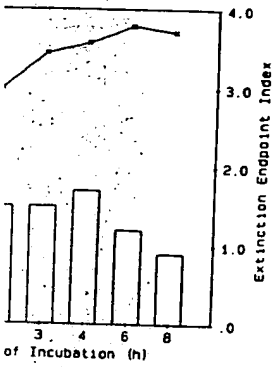


Fig. 2. Organism viability and supernatant toxicity of 4 different strains of *P. haemolytica* over an 8-h period. Viability measurements (line graphs) are the mean number of colony forming units per milliliter performed in triplicate on duplicate samples of a single culture. Toxicity measurements (bar graphs) are represented by an extinction endpoint index = log of the reciprocal of a mean extinction endpoint from the supernatants of duplicate cultures. Note discontinuity in duration of incubation scale at 4 h. (A) Serotype 1; (B) Serotype 2; (C) Serotype 9; (D) untypable strain. Dotted lines in (D) indicate that the viability at 6 and 8 h was $< 1 \times 10^7$ ml^{-1} (the lower limit of detection used).

were the absence of serologic response to the S9 organism for calves vaccinated with the S5 and S6 strains, and the serologic response of each calf was generally lower to the UT strain. With the exception of the calves vaccinated with the S5 and S9 strains, there was no striking predilection of the sera to recognize the autologous serotypes. Sera from the calves vaccinated with the UT strain reacted poorly in the ELISA and had to be used at a lower dilution than the other sera so that an antibody response could be detected. In addition, sera from the UT vaccinates reacted at least as well to each of the remaining serotype organisms as to the autologous antigen.

Vaccination with any of the 6 serotype strains also resulted in an increase in



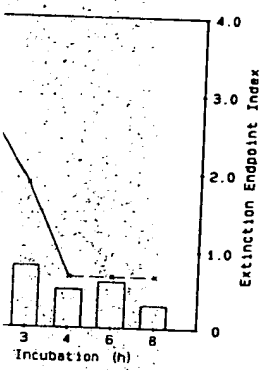


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TABLE II

Comparison of toxicity[a] of 6 different *P. haemolytica* strains for bovine peripheral blood leukocytes

Strain serotype	Average CFU ml^{-1} [b] ($\times 10^8$)	Bacterial dilution[c]			
		10^{-1}	10^{-2}	10^{-3}	10^{-4}
S1	2.8	2195 ± 188 (100.0)	1635 ± 146[f] (66.0)	1143 ± 71[f] (21.8)	962 ± 58 (5.5)
S2	2.3	2031 ± 87[e] (100.0)	1674 ± 136[f] (69.5)	1249 ± 116 (31.3)	979 ± 47 (7.0)
S5	2.6	2268 ± 137 (100.0)	1892 ± 99 (89.1)	1280 ± 104 (34.1)	1010 ± 71 (9.8)
S6	3.2	2132 ± 71 (100.0)	1718 ± 146[f] (73.5)	1228 ± 91 (29.4)	969 ± 32 (6.1)
S9	1.8	2279 ± 88 (100.0)	1985 ± 79 (97.5)	1282 ± 35 (34.3)	1028 ± 50 (11.4)
UT[d]	3.8	2230 ± 88 (100.0)	1960 ± 101 (95.2)	1280 ± 65 (34.1)	986 ± 24 (7.6)

[a]Toxicity is expressed as counts per 4 min of ^{51}Cr released from labelled leukocytes after exposure to the organisms. Values represent the mean of 10 counts from duplicate assays each prepared in pentuplicate. Numbers in parentheses represent the percentage killing of the leukocytes calculated from the mean count compared to a total release control (4 N NaOH).

[b]Average number of colony forming units per milliliter in undiluted bacterial suspensions as determined by duplicate plate counts.

[c]Serial 10-fold dilutions were prepared from bacterial suspensions standardized to a common optical density.

[d]Untypable strain.

[e]Value is significantly lower ($P < 0.05$) than all except S6.

[f]Value is significantly lower ($P < 0.05$) than others at the same dilution.

antibody response to a *P. haemolytica* S1 carbohydrate–protein subunit for all but one of the 18 cattle injected (Table IV). The mean increase in response after 2 injections was ~2.1–2.7 times higher for sera from animals vaccinated with the S1 strain than for sera from animals vaccinated with all other serotype strains except S6.

Production of serum antibodies which could neutralize leukotoxin from each of the strains was induced by vaccination with any of the 6 strains studied. However, the group mean titers varied widely (Table V). Group mean LN antibody titers at Day 0 against S1 strain leukotoxin ranged from 2.0 to 9.5 (data not shown). If for each type of leukotoxin neutralized, the vaccination groups are arranged in order of decreasing mean titer, the order is almost identical regardless of the serotype of the leukotoxin source. Thus, the mean LN titer for the group vaccinated with the S1 strain was highest against each leukotoxin preparation, follwed in order by the means for the groups vaccinated with S9, S2, S6, S5 and the UT strain. If within each vaccination group, the

TABLE III

Antibody responses of cattle to each *Pasteurella haemolytica* serotype as measured by an enzyme-linked immunosorbent assay

Vaccination	Sampling time	Antigen[a]					
		S1	S2	S5	S6	S9	UT
S1	Day 0	0.07 ±0.03[b]	0.08±0.04	0.07±0.03	0.08±0.05	0.09±0.02	0.02 ±0.01
	Day 21	0.38 ±0.11	0.35±0.10	0.49±0.18	0.40±0.10	0.20±0.04	0.13 ±0.05
S2	Day 0	0.05 ±0.04	0.03±0.01	0.06±0.04	0.06±0.04	0.07±0.04	0.04 ±0.02
	Day 21	0.30 ±0.13	0.41±0.12	0.37±0.16	0.29±0.12	0.19±0.07	0.19 ±0.07
S5	Day 0	0.01 ±0.006	0.02±0.007	0.08±0.02	0.01±0.01	0.01±0.01	0.001±0.00
	Day 21	0.23 ±0.04	0.22±0.003	0.40±0.08	0.25±0.09	0.08±0.01	0.10 ±0.02
S6	Day 0	0.01 ±0.01	0.01±0.01	0.02±0.01	0.01±0.01	0.01±0.004	0.01 ±0.005
	Day 21	0.26 ±0.10	0.21±0.09	0.29±0.13	0.26±0.09	0.06±0.04	0.10 ±0.06
S9	Day 0	0.005±0.002	0.01±0.004	0.01±0.008	0.01±0.01	0.04±0.02	0.02 ±0.02
	Day 21	0.19 ±0.10	0.21±0.09	0.23±0.10	0.21±0.09	0.48±0.15	0.16 ±0.08
UT	Day 0	0.08 ±0.03	0.12±0.02	0.11±0.03	0.11±0.04	0.29±0.10	0.05 ±0.03
	Day 21	0.27 ±0.06	0.36±0.15	0.30±0.13	0.34±0.15	0.43±0.11	0.28 ±0.12

[a]Formalin-killed *P. haemolytica* equilibrated spectrophotometrically such that there were 1×10^9 CFU equivalents ml^{-1}.
[b]Results are expressed as the mean absorbance$_{490}$ ± standard deviation. Sera from cattle vaccinated with S1, S2, S5, S6 and S9 were diluted 1:250. Sera from cattle vaccinated with UT were diluted 1:100.

TABLE IV

Antibody responses for calves vaccinated with 6 strains of *Pasteurella haemolytica* to a carbohydrate-protein subunit from serotype 1 organisms

Vaccination strain serotype	No. of calves	Mean antibody response[a]		
		Day 0	Day 21	Increase[b]
S1	4	0.27±0.11	0.75±0.25	0.49
S2	3	0.46±0.15	0.64±0.25	0.18[c]
S5	2	0.36±0.05	0.59±0.15	0.23
S6	3	0.21±0.06	0.62±0.13	0.41
S9	3	0.21±0.08	0.44±0.06	0.23
UT[d]	3	0.20±0.03	0.38±0.09	0.18

[a]Mean absorbance$_{490}$ (±SD) in an enzyme-linked immunosorbent assay.
[b]Increase in group mean absorbance$_{490}$ from Day 0 to Day 21.
[c]Serum from one of the 3 animals showed no increase.
[d]Untypable strain.

TABLE V

Mean neutralizing antibo
kotoxin produced by each

Vaccination strain serotype	No. of calves
S1	4
S2	3
S5	2
S6	3
S9	3
UT[c]	3

[a]Geometric mean tite
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[b]Leukotoxins from e
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[c]Untypable strain.

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TABLE V

Mean neutralizing antibody titers for cattle injected with 6 strains of *P. haemolytica* against leukotoxin produced by each strain

Vaccination strain serotype	No. of calves	Mean neutralizing antibody titers (±SD)[a] against leukotoxin from serotypes[b]					
		S1	S2	S5	S6	S9	UT[c]
S1	4	107.6 ±1.8	1448.0 ±2.8	107.6 ±1.8	64.0 ±1.6	45.2 ±1.4	362.0 ±1.4
S2	3	80.6 ±1.9	203.2 ±2.4	64.0 ±1.8	40.3 ±1.7	32.0 ±1.8	203.2 ±1.4
S5	2	32.0 ±4.0	90.5 ±5.7	32.0 ±4.0	32.0 ±4.0	16.0 ±4.0	128.0 ±4.0
S6	3	50.8 ±1.4	128.0 ±2.7	40.3 ±1.9	25.4 ±2.4	20.2 ±1.9	128.0 ±1.8
S9	3	64.0 ±1.8	256.0 ±0	80.6 ±1.4	50.8 ±1.9	40.3 ±1.4	256.0 ±1.8
UT[c]	3	12.7 ±1.4	64.0 ±1.8	16.0 ±0.0	8.0 ±0.0	8.0 ±1.8	40.3 ±1.4

[a]Geometric mean titers (±SD) for sera collected on Day 21 expressed as the reciprocal of the last serum dilution that neutralized the leukotoxin.
[b]Leukotoxins from each serotype were standardized to an extinction endpoint of 1/16 before use in the assay.
[c]Untypable strain.

leukotoxin-producing serotypes are arranged in order of decreasing mean titer, the order again is almost identical. Thus, the mean titer within each vaccination group is highest against leukotoxin from the S2 strain, followed in order by toxins from the UT strain and then S1, S5, S6 and S9.

DISCUSSION

The organisms used in the study were indistinguishable in culture, with the exception of the very mucoid appearance of the S6 strain. An assumption that the mucoid nature of the colonies might indicate a greater abundance of capsular material on these organisms was not supported microscopically in that all of the strains appeared to be similarly encapsulated. The demonstration of encapsulation of the UT strain strengthens the suggestion made by Frank (1980) and by Donachie et al. (1984) that at least some of the IHA-negative strains of *P. haemolytica* are not untypable due to a deficiency in soluble IHA antigens, but are rather antigenically distinct from the established IHA-positive serotypes. Throughout the study, suspensions of the S9 strain had to be standardized to a greater absorbance at 650 nm in order to provide cultures with numbers of viable organisms equivalent to those for the remainder of the

strains. This may be due, at least in part, to the differences in shape and size of the organisms.

When injected into the lungs of healthy cattle, the lesion scores produced by the S2, S5, S6 and S9 strains were comparable to or exceeded that produced by the S1 strain. By contrast, the UT strain produced only minimal lung lesions, indicating less pathogenic potential for the UT strain in vivo. In an in vitro assay, each of the strains was toxic for bovine mixed peripheral blood leukocytes. None of the strains was found to be significantly more or less toxic than the others at a majority of the dilutions tested ($P > 0.05$). Similarly, several serotypable strains of *P. haemolytica* were found in earlier studies to have similar toxicities for bovine and sheep alveolar macrophages (Benson et al., 1978; Sutherland and Donachie, 1986). That the UT strain was as toxic in the in vitro assay as the remainder of the organisms indicates that its inability to produce lesions in vivo may have been due to a deficiency in some pathogenic parameter other than leukotoxin production. In a recent study (Chang et al., 1987), living organisms and concentrated culture supernatants from bacterial strains representing each of the 15 recognized serotypes of *P. haemolytica* were found to be toxic for bovine neutrophils, whereas 4 UT strains were not toxic either as culture supernatants or as living bacterial suspensions. The UT strains used in that study, however, were isolated from chickens rather than from ruminants and the appropriateness of their inclusion as *P. haemolytica* isolates was questioned.

Although each serotype strains produced leukotoxin in 1-h cultures in medium supplemented with 7% FBS, the toxins produced were not equal in total toxic activity. The S9 strain consistently produced leukotoxin with extinction endpoints 1–2-fold higher than those produced by the S1, S5 and S6 strains, even though the percentage increase in viable counts was similar for all 4 cultures. By contrast, the S2 and UT strains consistently produced toxins with extinction endpoints 1–2-fold lower than those produced by the S1, S5 and S6 strains. In addition, the increase in number of viable organisms during the incubation period was lower for the S2 and UT strains than for the other 4 cultures. The ability of single strains of *P. haemolytica* from serotypes 1–12 to produce leukotoxin to varying degrees has been shown previously (Shewen and Wilkie, 1983). Differences in leukotoxin production in that study did not appear to correlate with the differences shown in the present study. However, the 2 studies utilized different bacterial strains, different types of bovine indicator cells and different systems for measuring degree of toxicity.

To evaluate the relationships between leukotoxin production and bacterial viable counts, cultures of the S1, S2, S9 and UT strains were evaluated for these parameters during an 8-h period. Serotypes 5 and 6 were not studied by this technique because they produced leukotoxin concentrations similar to S1. The viability and toxicity profiles for the S1 and S2 cultures were similar except that S2 had a 2-h lag phase relative to the S1 culture. Each strain had a

rise in toxicity in association with an increase in viable organisms, followed by a decline in toxicity as the organisms reached the stationary phase. These results are consistent with an earlier study (Shewen and Wilkie, 1985) in which it was determined that a *P. haemolytica* S1 culture produced leukotoxin only during the lag and logarithmic growth phases of growth. The results of the 8-h culture experiments indicate that the low toxicity of supernatants from 1-h cultures of S2 is primarily due to a prolonged lag phase for this particular strain compared to that for the S1 strain.

Leukotoxin production by the S9 and UT strains was also correlated with growth of the organisms during the first hour of incubation. The subsequent decline in viability for both of these cultures cannot be explained. The concomitant decrease in toxicity of the UT culture supernatant was similar to that seen for the S1 and S2 cultures. By contrast, the S9 culture supernatant remained highly toxic, despite a rapid decrease in culture viability. One possible explanation for this phenomenon is that the leukotoxin from this organism is more stable at 37°C than those produced by the other strains studied.

All cattle in the vaccination trial produced leukotoxin-neutralizing antibodies regardless of which strain was used for injection. Marked differences occurred in the mean LN titers among the vaccination groups, with the S1 strain being the most effective immunogen, followed by the S9 and S2 strains. In cross-neutralization studies, it was determined that there was no strain specificity shown with regard to type of leukotoxin neutralized. It has been reported previously that rabbit sera produced against *P. haemolytica* strains from serotypes 1 to 12 could neutralize toxin from an S1 strain, with the exception of the serum produced against S5 (Shewen and Wilkie, 1983). In that study, the type-specific antisera tended to neutralize their homologous toxins more effectively than the heterologous preparation. Perhaps the lapine system is more discriminatory than the bovine system towards subtle differences in the leukotoxin antigen preparations used.

Cross-reaction was seen also in the antibody response of vaccinated calves to somatic antigens from each of the organisms. A notable exception was the poor reaction of sera from calves receiving the UT strain to the serotypes. In that case, antibodies were detected only when sera were 2.5 times more concentrated than sera from the calves vaccinated with the serotypes. These results again support the observed inherent differences between the UT strain and the other serotype strains studied.

It has been suggested that LN antibodies may be important in protection againsy bovine pneumonic pasteurellosis, whereas antibodies to bacterial surface components do not necessarily correlate with protection (Wilkie, 1982; Gentry et al., 1985b; Chang et al., 1986). It was further suggested that opsonizing antibodies may even enhance severity of the disease process by increasing bacterial induced cytotoxicity of phagocytes (Markham and Wilkie, 1980). If these observations are correct, an organism such as the S9 strain used in the

present study might have potential as a vaccination strain. Inoculation with the S9 organism produced the lowest serum antibody responses for any group to somatic antigens from the S1 strain. Similarly, sera from the S9 vaccinates reacted poorly to an extract antigen from the S1 strain while maintaining a relatively high leukotoxin-neutralizing antibody response.

Pasteurella haemolytica S2 is a common nasal isolate from healthy cattle (Frank, 1985). Although the S2 strain used in the present study was shown to be toxic for bovine leukocytes and pathogenic for a calf when introduced directly into the lungs, its performance in the 8-h toxin production experiment would suggest that it might be a less efficient pathogen than the S1 organism. It was suggested that the pathogenic process in pneumonic pasteurellosis consists of a build up of organisms in the lung to a toxic concentration (Smith, 1960). In the present study, the S2 strain grew somewhat slower than the S1 strain in serum-supplemented medium. Slower growth and consequent lower leukotoxin production in vivo might allow host defenses to combat an infection before the organism increased to a threshold level that would cause disease. The difference in growth rates, if borne out among other S1 and S2 strains, could also explain the shift reported by Frank and Smith (1983) in the prevalence of isolation from S2 to S1 after cattle are transported from the farm to the feedyard.

The S9 and UT strains both failed to maintain logarithmic phase growth in the 8-h toxin production experiments. All other parameters measured in this study indicated that the S9 strain might have particular pathogenic potential. Its outstanding toxin-producing capabilities make it especially interesting for further study. By contrast, the UT strain appeared to be one of the least potentially pathogenic and immunogenic of the 6 organisms examined. Although the group of *P. haemolytica* organisms classified as untypable is undoubtedly heterogeneous and the strain used in the present study may not necessarily be "representative", other investigators have also suggested that untypable strains may not be as pathogenic as those in the established serotypes (Donachie et al., 1984; Chang et al., 1987).

In conclusion, all 6 of the strains used in this study appeared to be potential pathogens. Each was encapsulated and produced leukotoxin, with toxins from the S5, S6 and S9 strains having as much or more total toxic activity as that from the S1 strain. Furthermore, each of the other strains was at least as toxic as the S1 strain for bovine leukocytes in an in vitro assay and all but the UT strain produced comparable lesions in vivo. These results seem to support the recent study in which the pathogenicity of 4 different *P. haemolytica* serotypes was compared in vivo in sheep (Gilmour et al., 1986). These authors concluded that there was no significant difference in the ability of their serotype strains to produce disease and that differences in the frequency of isolation of those serotypes in field outbreaks was not due to differences in virulence of the organisms. However, subtle differences in growth rates or ability to maintain

stationary phase in vitro as shown in the present study, may indicate a selective disadvantage for certain strains of *P. haemolytica* for disease production.

Although the organisms used in the present study were single isolates from their respective serotypes and may not necessarily be representative of the serotypes as a whole, the authors feel that comparison of additional serotype strains to organisms from S1 are important in helping to understand the almost exclusive prevalence of S1 organisms in pneumonic pasteurellosis. Continued studies on bovine isolates would also be useful in determining important pathogenic mechanisms and protective antigens associated with *P. haemolytica* S1.

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